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Study of Mutations in *kat*G and *inh*A Genes by High Resolution Method in Isoniazid Resistant *Mycobacterium tuberculosis* Isolates

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Background: Tuberculosis is one of the infectious diseases worldwide that has resurgence by the AIDS epidemic and led to the rise of drug-resistant tuberculosis patients. Thus, it seems essential to monitor the drug susceptibility in tuberculosis patients. The new High Resolution Melting (HRM) method is simple, rapid and inexpensive for detection of the mutations responsible for drug resistance in *Mycobacterium tuberculosis* isolates. In this study, we used HRM method to detect mutations in samples collected from tuberculosis patients.
Materials and Methods: Three thousand sputum samples were collected from patients with suspected tuberculosis referred to Iran Remedial Center over a period of 2 years, out of which 2000 samples were found positive for *M. Tuberculosis* on direct smear. After extraction of genomic DNA from sputums, HRM method was used to detection of mutations in *kat*G and *inh*A genes.
Results: Our findings showed that 120 out of 2000 positive smear samples were resistant to isoniazid due to mutations in *kat*G and *inh*A genes, out of which, 25 mutation was found in *inh*A gene and 95 mutation in *kat*G gene.
Conclusion: The HRM method is quick, easy and affordable without need of culture and any post PCR process for diagnosing of drug resistance in tuberculosis clinical samples.

Keywords: Mycobacterium tuberculosis, Isoniazid, High Resolution Melting

1. Background

At the beginning of the 20th century, infectious diseases were the main cause of mortality in different societies and tuberculosis was one of the major diseases (1). Still, it is one of the most dangerous infectious diseases responsible for 3 million deaths worldwide each year (3), which is postulated to increase by the enhancement of drug-resistant bacteria and rise of epidemic AIDS patients (4). Therefore, it is necessary to control the infection. Since drug-resistant tuberculosis patients probably remain infectious for a long period (5, 6), drug-resistant tuberculosis has more serious consequences for the public health than those that are sensitive to the drugs (7, 8). Isoniazid is one of the main drugs that are used to treat tuberculosis (9). Its antibiotic activity depends on bacterial activation by catalase peroxidase (*kat*G) enzyme that creates active radicals in the *Mycobacterium tuberculosis* (10, 11). If mutation is caused in *inh*A, another target gene resistance to isoniazid is possible (12). The Enol-Acp reductase is an enzyme involved in mycolic acid synthesis and its interference disrupts cell wall biosynthesis (13). Thus, *kat*G and *inh*A are the most popular mutations associated with resistance to isoniazid, the drug which destroys the tubercle bacillus after starting therapy in a short period of time (14). Resistance to these drugs can be associated with incurable results (15). Considering the detection of resistance with old methods such as culture requires a long period of time and is sometimes associated with contamination (16). Thus, for rapid diagnosis of such type of resistance it is better to use molecular methods based on PCR (17). High resolution melting method is rapid, easy and economical for isolating genotypes without using specific probes (18) that are shown in HRM (High Resolution Melting) analysis of nucleic acid sequences with high accuracy based on the difference between the formed curves (19). This study was conducted to evaluate a quick and convenient method for the detection of mutations in isoniazid in sputums from patients referred to health and care centers in Masih Daneshvari tuberculosis center in Iran.

2. Objectives

In this study we aim to investigate the mutations in *kat*G and *inh*A genes in sputum from tuberculosis patients by the using of high resolution melting method.

3. Materials and Methods

3.1. Sample collection

This study was conducted on 3000 patients with suspected tuberculosis referred to health and care centers in Iran in the past two years.

3.2. Smear preparation

First collected sputum was homogenized and decontaminated using sodium and N-acetyl-L-cysteine. Then, the smears were examined after Ziehl Nelson staining for the presence of acid fast bacillus. Among samples, 2000 were found positive on direct smear examination and their sputum samples were stored at -20°C.

3.3. DNA extraction

Whole DNA was extracted using highly pure PCR template preparation kit from Roche (Roche, Ltd. Germany). After lysis and denaturing other proteins by suitable buffer, the samples were incubated at 56 °C. Lyses substances were added into the column and the samples were then rinsed to remove other components from environment. The obtained DNA was centrifuged with 95% cold ethanol for 10 min at the 14000 rpm. Supernatant was removed from the sample and the DNA sample was rinsed with 70% cold ethanol. After centrifugation, ethanol was removed and washed DNA was obtained. The DNA was

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then air dried and dissolved in 70 µl buffer provided with the manufacturer's kit.

3.4. Primer design and PCR reaction conditions

Two pairs of forward and reverse primers were designed for *kat*G and *inh*A genes, that had polymorphism for the presence of mutation (Table 1). PCR was performed as follows: 5µl genomic DNA was added to the mixture of 2.5µl 10x PCR buffer, 1µl from (50 µM) $MgCl_2$, 1µl from each primer (10 $pmol\mu l^{-1}$), 1µl dNTP (10µM) and Taq polymerase (0.4µl). The total volume of reaction mixture was 25µl. The PCR reaction conditions were the same for both genes and included in initial denaturation of 95°C for 10min and 40 cycles of 95°C for 30sec, 55°C for 30sec and 72°C for 40sec.

Table 1. Primers designed to amplify genes resistant to isoniazid

Primer name	Gene	Primer sequence	Amplicon size (bp)
Tb92-F Tb92 -R	*inh*A	5´-CCTCGCTGCCCAGAAAGGGA-3´ 3´-ATCCCCCGGTTTCCTCCGGT-5´	209
Tb86-F Tb86 -R	*kat*G	5´-GAAACAGCGGCGCTGATCGT-3´ 3´-GTTGTCCCATTTCGTCGGGG-5´	248

3.5. High resolution melting analysis

HRM curve analysis was performed on Rotor Gene 6000 device. Once PCR amplification was completed, melting curve data was analyzed using initial stage holding at 60°C for 30 sec. Temperature changes were examined with Rotor Gene 6000 (Corbett Life Science Pty., Ltd). Rotor Gene software examined nucleotide differences samples in three forms of normalized graphs; graph with variations and melting curve (Table 2).

Table 2. Temperature conditions of HRM reaction

Cycle	Cycle Point
Hold 1	at 95°C for 7 min 0 sec.
Cycling (40 Repeats)	Step 1; at 96°C, hold 30 sec. Step 2; at 62°C, hold 30 sec, Step 3; at 68°C, hold 45 sec.
Hold 2 Hold 3 Hold 4	at 68°C, 7 min 0 sec. at 50°C, 5 min 0 sec at 75-95°C 5min on the 1st step,

3.6. DNA sequencing

Samples were selected randomly from analyzed groups by Rotor Gen 6000. After PCR, we used the purified DNA as the template for sequencing in a BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA). Then we heated samples up to 96°C for 1 min and then ran for 35 cycles of 96°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min. Ethanol precipitation method described in the manufacturer's manual was used to purify The extension products . The pellet was rehydrated in 15µl of formamide, heat denatured at 95°C for 5min in a thermal cycler, and immediately put on ice for 5min. The samples sequenced with the BigDye Terminator kit were electrophoresed on an ABI Prism 3130 instrument (Applied Biosystems).

4. Results

4.1. Resistance to isoniazid and evaluation of katG and inhA

HRM analysis was used for 2000 positive smear samples to evaluate 209 bp fragment of *kat*G gene and 248bp fragment of *inh*A gene. By examining these genes, samples were placed in different groups for analysis, based on differences in melting point and shape of curves. Graph differences were probably due to nucleotide differences in the samples. Normalized graph**,** Difference graph and Melting curve of sector 209 from *kat*G gene is shown in Figure 1 and Normalized graph**,** Difference graph and Melting curve of sector 248 from *inh*A gene is shown in Figure 2. Each reproduced curve represents one of the samples that were placed in three different groups.

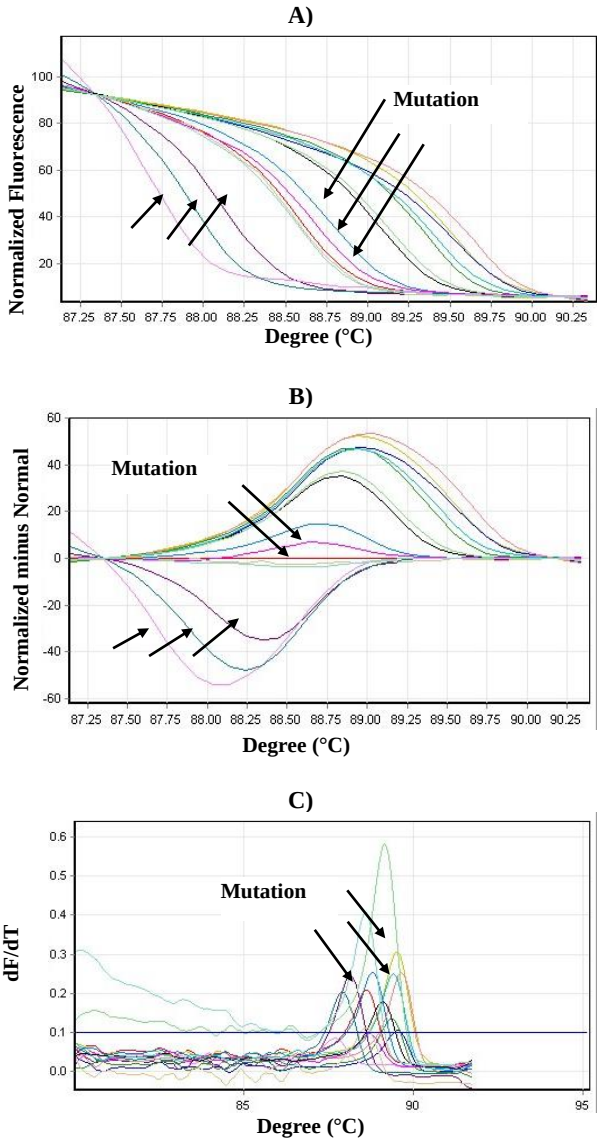

Figure 1. Mutation in 209bp fragment of *kat*G gene. A) Normalized graph; B) Difference graph; C) Melting curve of sector 209 from *kat*G gene. Each reproduced curve represents one of the samples that were placed in three different groups.

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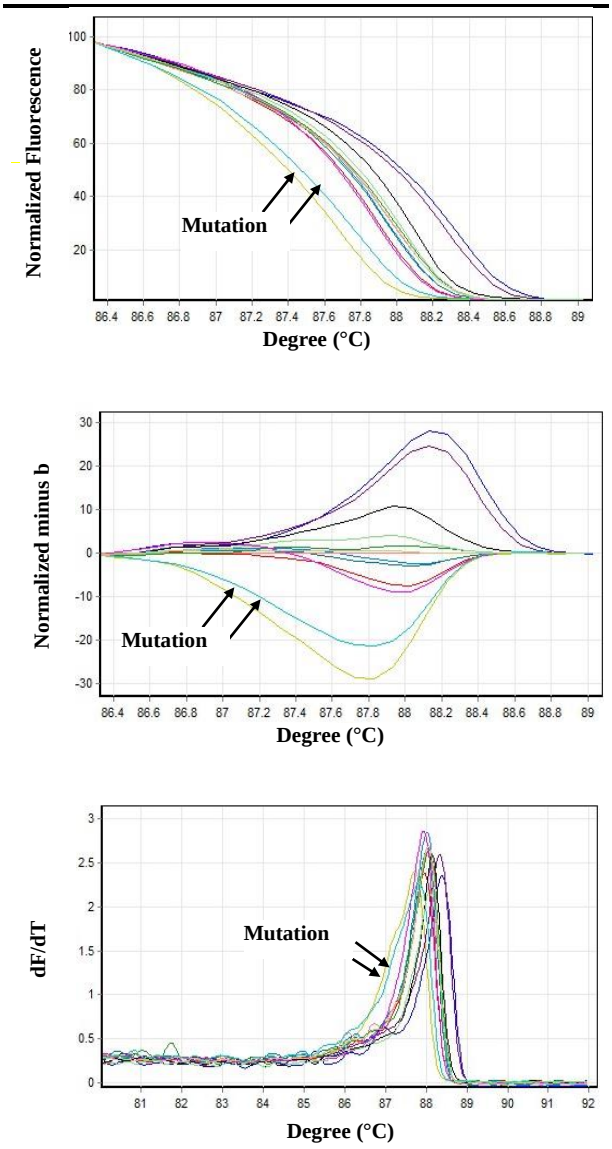

Figure 2. Mutation in 248bp fragment of *inh*A gene.

In order to verify the results and to ensure the nucleotide differences in samples and the identification of the wild and mutated groups considered the interested regions, the samples from each group were sequenced. On the analysis of 2000 samples, they were classified in three different groups based on difference in their melting points. The samples that had the same melting point or similar curve were placed in one group.

4.2. Sequencing results for katG gene

Samples of three different groups were sequenced for 209bp product of *kat*G gene based on HRM analysis. According to sequencing results, two groups of samples had mutated sequence and one group had normal sequence. According to the sequencing process, 95 samples were placed in two mutated groups and 1905 samples were placed in normal sequence group. Among 95 mutated samples, 75 samples (71.25%) showed a mutation in codon 315 of *kat*G gene and 20 samples (19%) showed mutation in codon 279 of the same gene. Among 75 samples which had a mutation in codon 315 KatG gene, 55 samples (41.25%) (Ser→Thr) AGC→ACC, 15 samples (11.25%) (Ser→Ile) AGC→ATC, 3 samples (2.25%) (Ser→Asn) ACC→AAC and 2 samples (1.5%) (Ser→Thr) AGC→ACA.

Following changes were carried out in 20 samples that had mutation in codon 279 from *kat*G gene: 14 samples (2.8%) (Gly→Thr) GGC→ACC, 4 samples (0.8%) (Gly→Arg) GGC→CGC, 2 samples (0.4%) (Gly→Ile) GGC→ATC.

*4.3. Sequencing results for inh*A *gene*

Samples from three different groups were sequenced for sector 248from *inh*A gene based on HRM analysis. The sequencing results for this gene showed that two groups of investigated samples had normal sequence and one group had mutated sequence. From 2000 samples, only 20 samples had mutations. All 20 mutated samples responsible for resistance to isoniazid in *kat*G and *inh*A genes are given in Table 3. Among 2000 investigated samples, 75 samples with mutation in *kat*G gene and 20 samples in *inh*A gene had resistance to isoniazid.

Table 3. Mutations found in the *Kat*G and *inh*A genes by Silver sequence

Analyzed gene region	Location of mutation	Nocleotide Change	Amino Acid Change	No of strains (%)
*kat*G	315	AGC→ACC AGC→ATC AGC→AAC AGC→ACA	ser→Thr ser→Ile ser→Asn ser→Thr	55 15 3 2
*kat*G	279	GGC→ACC GBC→CGC GGC→ATC	GLY→Thr GLY→Arg GLY→Ily	14 4 2
*inh*A	15th locus	C→T	C→T	20

5. Discussion

According to the national TB directions (20), there are many different factors that may cause drug-resistant tuberculosis, including: patient being uninformed of free treatment for the disease, ignorance of patients about the disease, factors related to the drug such as unsuitable quality and dosage of medication and failure to educate patients and their families (21). In 2005, about 260,000 cases of total MDR were reported and in some areas up to 10% prevalence of extensive drug resistance (XDR) were reported (19). Providing methods for determining the amount of sensitivity seems necessary after the correct diagnosis of bacterial infections (22). Understanding the genetic basis of drug resistance will help to develop effective methods for the quick determination of drug-resistant *M. tuberculosis* (18). The aim of our study was to investigate mutation in *kat*G and *inh*A genes in tuberculosis patients sputums and also to identify the most common mutations in these genes with the evaluation of high resolution melting method which is a simple, rapid and standard method without the need for post PCR procedures, specific probes and long period of time for culture (12). Using of efficient methods provide fast detection of disease in patients and also prevent of infection transmission to other people. In contrast to previous studies carried out by researchers, we directly used sputum samples to purify DNA without the use of culturing techniques. Also, we examined common mutations in the sequence of TB86 and TB92 and showed that HRM techniques have this ability to identify all of the mutations without the use of culture. Conventional PCR needs to post PCR step that it causes much more contamination. Additionally, real time PCR methods need specific probes that spend more times whilst more currency. While, in the previous studies culturing technique was used to evaluate known resistant and sensitive samples. According to this study,

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among 3000 examined samples, 120 samples were found resistant to isoniazid. Of these, 95 samples had mutations in codon 315 and 279 in *kat*G gene and 25 samples had mutations in the C→T (15th area) in *inh*A gene. In a study conducted by Aslan and colleagues (2008), HRM method was used for the detection of MTB resistance to isoniazid and rifampin (23). The study was carried out to identify specific mutations using resistant and sensitive cultured samples with mutation in S315T and D310A areas in *kat*G gene, and mutation in the C→T 15th area was selected to evaluate the resistance to isoniazid (21). Thus, all previous studies used PCR techniques, culture, RFLP or real time PCR for examining known resistant and sensitive samples. HRM method is a very convenient and sensitive molecular method for screening pointed mutations in clinical samples with sensitivity (98.6%) and specificity (100%) (21). Since this technique is fast, inexpensive and amenable to a large number of samples and doesn't require further processing of samples, it can be suggested for drug-resistant TB patients as the easiest way to check and detect pointed mutations (12). Another advantage is that the procedure is performed in the same tube without any post-PCR processing, thus it is useful for routine diagnostic testing without any contamination. In the present study, the SYTO dyes were used which is more useful for HRM application compared with other colors. Also, understudy samples showed that the most common mutations associated with drug-resistance to isoniazid were in agreement with previous studies.

6. Conclusion

We conclude that a broader study with more samples of the types of mutations associated with tuberculosis drug-resistance should be investigated.

Conflict of Interests

The authors declare they have no conflict of interests.

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Authors' Contributions

Zoha Tavakoliamol performed the experiments, analyzed data and wrote the manuscript, Ali Nazemi Conceived, designed the experiments and analyzed data.

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